

# Effects of Hypoxia and Hypercapnic Hypoxia on the Localization and the Elimination of *Vibrio campbellii* in *Litopenaeus vannamei*, the Pacific White Shrimp

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**Abstract.** Low oxygen (hypoxia) and elevated CO<sub>2</sub> (hypercapnia), are characteristic of estuarine environments. Although hypoxia and hypercapnic hypoxia decrease the resistance of shrimp to bacterial pathogens, their direct effects on the immune system are unknown. Here we present evidence demonstrating in the penaeid shrimp *Litopenaeus vannamei* that both hypoxia and hypercapnic hypoxia affect the localization of bacteria, their conversion from culturable to non-culturable status (bacteriostasis), and their elimination from hemolymph and selected tissues. Shrimp were injected with a sublethal dose of a pathogenic strain of *Vibrio campbellii* expressing green fluorescent protein and resistance to kanamycin. Real-time polymerase chain reaction was used to determine the number of intact *V. campbellii* in hemolymph, gills, hepatopancreas, heart, and lymphoid organ. Selective plating was used to quantify the injected bacteria that remained culturable. We found that both hypercapnic hypoxia and hypoxia increased the percentage of culturable bacteria recovered from the hemolymph and tissues, suggesting an overall decrease in bacteriostatic activity. Hypoxia and hypercapnic hypoxia generally increased the distribution of intact *V. campbellii* to the hepatopancreas and the gills, which are major targets for the pathogenic effects of *Vibrio* spp., without affecting the number of intact bacteria in the lymphoid organ, a main site of bacterial accumulation and bacteriostatic activity.

## Introduction

Penaeid shrimp are often exposed to large fluctuations in dissolved oxygen that are characteristic of the estuarine environments in which they live. Globally, the severity of hypoxia in coastal waters is increasing at a drastic rate (Diaz and Rosenberg, 1995; Diaz, 2001). In addition, hypoxia often co-occurs with increased levels of CO<sub>2</sub> (hypercapnia) produced by respiration, causing a decrease in water pH (Cochran and Burnett, 1996; Burnett, 1997; Ringwood and Keppler, 2002). Although not always the primary cause of mortality, hypoxia and hypercapnic hypoxia decrease the resistance of shrimp to bacterial pathogens. Hypoxia ( $P_{O_2} = 3.1$  kPa,  $1$  mg O<sub>2</sub> l<sup>-1</sup>) decreased the survival of *Penaeus stylirostris*, the black tiger shrimp, following injection of the bacterial pathogen *Vibrio alginolyticus* (Le Moullac *et al.*, 1998). Hypercapnic hypoxia ( $P_{CO_2} = 1.8$  kPa;  $P_{O_2} = 4$  kPa) increased mortality in the shrimp *Litopenaeus vannamei* and *Palaemonetes pugio* injected with the bacterial pathogen *V. parahaemolyticus* (Mikulski *et al.*, 2000). The specific effects of oxygen and pH on the susceptibility of shrimp to these invading bacterial pathogens remain unclear, but may involve changes in mechanisms of immune defense such as the number of circulating hemocytes, the production of reactive oxygen species (ROS), and the activity of phenoloxidase (Le Moullac *et al.*, 1998; Mikulski *et al.*, 2000; Cheng *et al.*, 2002). In addition, other physiological responses to hypoxia and hypercapnia, such as changes in cardiac stroke volume and heart rate and the redistribution of hemolymph flow (McMahon and Burnett, 1990; Burnett 1992, 1997), may alter the access of bacterial pathogens to tissues involved in disease or immune defense (see Discussion).

When introduced by injection, bacteria not trapped at the injection site quickly appear in the hemolymph and other

Received 9 December 2004; accepted 29 March 2005.

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**Abbreviations:** ANOVA, analysis of variance; CFU, colony-forming unit; HS, Holm-Sidak method for pairwise multiple comparison tests; PCR, polymerase chain reaction; ROS, reactive oxygen species.

tissues of crustaceans, but are rapidly eliminated (e.g., Smith and Ratcliffe, 1980; White and Ratcliffe, 1982; Clem *et al.*, 1984). Under well-aerated, normoxic conditions ( $P_{O_2} \approx 20$  kPa), penaeid shrimp also quickly remove bacteria from their hemolymph (Martin *et al.*, 1993; Burgents *et al.*, 2005). Suppression of pathogen growth, as detected by culture on artificial media, occurs much more rapidly than the removal of bacterial degradation products, as monitored by a variety of other techniques, including histology, radiolabel tracing, or real-time PCR (Martin *et al.*, 1993; van de Braak *et al.*, 2002; Burgents *et al.*, 2005).

Exposure to hypoxia or hypercapnic hypoxia decreases the rate at which culturable bacteria are eliminated from the hemolymph in a number of crustaceans. When *Penaeus monodon* was exposed to hypoxia ( $P_{O_2} = 5.4$ – $6.4$  kPa,  $1.8$ – $2.0$  mg  $O_2$   $l^{-1}$ ), the shrimp had significantly higher numbers of culturable *Vibrio harveyi* in their hemolymph 30 min after bacterial injection than did animals in well-aerated water (Direkbusarakom and Danayadol, 1998). Similar results were obtained when the freshwater prawn *Macrobrachium rosenbergii* was exposed to hypoxia (Cheng *et al.*, 2002). Holman *et al.* (2004) observed a significant increase in the number of culturable *Vibrio campbellii* that remained in the hemolymph of *Callinectes sapidus*, the blue crab, in hypercapnic hypoxia ( $P_{CO_2} = 1.8$  kPa;  $P_{O_2} = 4$  kPa) compared with animals held in normoxic conditions. In these studies, the increased retention of culturable bacteria in hemolymph that is associated with hypoxia and hypercapnic hypoxia could be the result of a decrease in the inactivation of culturable bacteria, a decrease in the physical removal of bacteria from the hemolymph, or a combination of both mechanisms.

Bacteria that accumulate in a variety of tissues are subsequently eliminated, although their rates of accumulation, conversion to non-culturable status (bacteriostasis), and degradation differ as a function of the crustacean host, the bacterial pathogen, and the tissue examined. Penaeid shrimp, unlike most crustacean species, possess a lymphoid organ that is apparently involved in the elimination of foreign material (reviewed by van de Braak *et al.* (2002). Burgents *et al.* (2005) quantified the bacteriostatic activity of four tissues of the penaeid shrimp *Litopenaeus vannamei* and found that the lymphoid organ was the main site of active uptake and bacteriostasis of pathogenic *V. campbellii* injected intramuscularly. Although the gills and the hepatopancreas were also major sites of bacterial accumulation, these tissues had lower levels of bacteriostatic activity (Burgents *et al.*, 2005). To clarify the role of each of these tissues in immune defense and to better understand how low oxygen and high carbon dioxide decrease the resistance of shrimp to bacterial pathogens, we examined the effects of hypoxia and hypercapnic hypoxia on the tissue accumulation and bacteriostasis of pathogenic *V. campbellii* injected into *L. vannamei*.

## Materials and Methods

### Animals

The present study was performed in conjunction with that described by Burgents *et al.* (2005). Individuals of *Litopenaeus vannamei* (Kona Specific Pathogen Free Stock, Oceanic Institute, Kona, HI) weighing between 5 and 9 g were obtained from Waddell Mariculture Center, Bluffton, South Carolina, and held at the Grice Marine Laboratory in Charleston, South Carolina. Shrimp were held in well-aerated recirculating seawater at 30 ppt salinity, 23–25 °C, and pH 7.8–8.1. They were fed daily with commercial shrimp pellets (Rangen Inc., Buhl, ID) and held for at least 2 weeks before use in experiments.

### Experimental design

We tested the influence of ambient pressures of oxygen and carbon dioxide on the tissue distribution and the fates of the bacterial pathogen *Vibrio campbellii* injected into the shrimp. In all treatments, shrimp injected with saline were used as controls. Shrimp were transferred from their holding tanks to 19-l glass aquariums in which concentrations of oxygen and carbon dioxide were regulated. Treatments consisted of different levels of ambient oxygen and carbon dioxide in the water in which the shrimp were held (Table 1). Shrimp were held in treatment conditions for 4 h before the injection of bacteria or saline as well as for the duration of the experiment (up to 4 h) after injection. The level of hypoxia ( $P_{O_2} = 4$  kPa) used in the present study is not lethal in *L. vannamei*, but it is below the critical oxygen pressure (5.6 to 6.9 kPa, unpubl. data) for the species — that is, the oxygen pressure below which the shrimp are not able to regulate their oxygen uptake.

Hypoxia and hypercapnic hypoxia were established by continuous gassing of water with either  $N_2$  (hypoxia) or a combination of  $N_2$  and  $CO_2$  (hypercapnic hypoxia). Specific oxygen levels were maintained by gassing the water intermittently with air. The oxygen pressure in the water was monitored using an oxygen electrode (YSI Model 58, Yellow Springs, OH), the output of which was fed into a data acquisition system (Sable Systems International, Las Vegas, NV). The data acquisition system was programmed to maintain a specific oxygen pressure by activating an aerator as

Table 1

Oxygen and carbon dioxide pressures in the three experimental treatments

Treatment	$P_{O_2}$ (kPa)	$P_{CO_2}$ (kPa)
Normoxia	19–20.6	<0.05
Hypoxia	4	<0.05
Hypercapnic hypoxia	4	1.8

needed. In the hypercapnia experiments, CO<sub>2</sub> pressures were held at 1.8 kPa by gassing the water constantly with 2% CO<sub>2</sub>. Normoxia was maintained by vigorous aeration with ambient air.

### Bacterial challenge

*Vibrio campbellii* 90-69B3 used in the present study were transformed with pMSB6, a plasmid that bears genes encoding green fluorescent protein (GFP) and resistance to the antibiotic kanamycin (kan), as described by Burgents *et al.* (2005). The injection dose was prepared as described by Burgents *et al.* (2005), and the shrimp were injected intramuscularly in the third abdominal segment with 2  $\mu\text{l g}^{-1}$  body weight of  $1 \times 10^7$  colony-forming units (CFU)  $\text{ml}^{-1}$  of *V. campbellii*, for a final injection dose of  $2 \times 10^4$  CFU  $\text{g}^{-1}$  shrimp. This bacterial dose is approximately one-tenth of the LD<sub>50</sub> for *L. vannamei* (Mikulski *et al.*, 2000) and was selected in order to profile a successful defense against the bacterial pathogen. At 15, 60, or 240 min after injection, hemolymph was sampled, and the gills, the heart, the lymphoid organ, the hepatopancreas, and the injection site (third abdominal segment) were dissected, weighed, and homogenized individually in 5 ml of sterile HEPES-buffered saline (2.5% NaCl, 10 mM HEPES; pH 7.5). Tissues were taken from 7–10 bacteria- or saline-injected animals for each treatment and time point; the numbers of tissues used in each statistical analysis (*n*) are given in Tables 2 and 3. Aliquots of the tissue homogenates were stored at  $-70^\circ\text{C}$  until they were used for real-time PCR, while the remainder of the homogenates were diluted and plated on selective plates.

Shrimp serving as saline controls were injected with equivalent volumes of sterile HEPES-buffered saline (2.5% NaCl; 10 mM HEPES; pH 7.5).

### Real-time polymerase chain reaction

Real-time PCR was used to quantify the total number of intact *V. campbellii* in the tissues of *L. vannamei*. Briefly, as described by Burgents *et al.* (2005), tissue homogenates were centrifuged at  $5,000 \times g$  and resuspended in distilled water. Samples were incubated at  $95^\circ\text{C}$ , and the resultant supernatants, containing the plasmids from lysed *V. campbellii*, were analyzed. A 129-bp fragment of the kanamycin-resistance gene was amplified using 200 nM forward primer RTKnF 5'-TGATGCGCTGGCAGTGTT-3', 200 nM reverse primer RTKnR 5'-CTCGCATCAACCAAACCGTTA-3', and 200 nM Taqman probe 5'-TGCGCCGGTTGCATTCGATTCCTGT-3', 5' labeled with 6-carboxyfluorescein (FAM) and 3' labeled with Black Hole Quencher-1 (Integrated DNA Technologies, Inc., Coralville, IA). Reaction mixtures (25  $\mu\text{l}$ ) were prepared using the QIAGEN QuantiTect Probe PCR Kit (catalog # 204343). The amplification was monitored using Applied Biosystem's 7000

Sequence Detection System and consisted of a 15-min incubation at  $95^\circ\text{C}$  to activate the hot-start polymerase, followed by 50 cycles of denaturation at  $95^\circ\text{C}$  for 30 s, annealing at  $56^\circ\text{C}$  for 45 s, and elongation at  $72^\circ\text{C}$  for 30 s.

Quantification using real-time PCR was limited to plasmids associated with intact *V. campbellii*. Detection of plasmids from lysed or degraded bacteria was not expected, due to the removal of the initial supernatant, as described above, and the short residence time of nucleic acids in the nuclease-rich environment of the tissue homogenate. As described by Burgents *et al.* (2005), water samples and tissue samples from saline-injected control shrimp were analyzed as negative controls to ensure that there was no amplification except that detected from the injected bacteria. Tissue samples from saline-injected control shrimp were also spiked with *V. campbellii* to test for any tissue-specific inhibition of the PCR. A standard curve was constructed on the basis of saline samples (2.5% NaCl, 10 mM HEPES; pH 7.5) with known numbers of CFU. The use of this standard curve allowed us to accurately compare the values obtained by real-time PCR and selective plating, since the number of injected bacteria would be the same whether enumerated by PCR or plating. Using this standard curve, we established that the real-time PCR could be used to quantify as few as one *V. campbellii* per 25- $\mu\text{l}$  reaction volume. Threshold cycles of replicate samples differed by no more than one cycle, and the efficiency of all reactions was greater than 1.80, with most above 1.90.

### Selective plating

Selective plating was used to quantify the number of culturable *V. campbellii* in each tissue. Homogenates were plated in marine agar supplemented with 1% NaCl and 100  $\mu\text{g ml}^{-1}$  kanamycin overlaid on thiosulfate-citrate-bile-sucrose (TCBS) agar supplemented with 2% NaCl and 100  $\mu\text{g ml}^{-1}$  kanamycin. Plates were incubated at  $25^\circ\text{C}$ , and the number of culturable *V. campbellii* in each tissue was quantified by counting CFU 24 h after plating. The selectivity of the plates was confirmed by plating the parent strain of the transfected *V. campbellii*, which does not contain the kanamycin-resistance gene, as a negative control. Water samples were plated to ensure that kanamycin-resistant *Vibrio* spp. were not present in the water in which the shrimp were held. Tissue homogenates from saline-injected shrimp were also plated to ensure that kanamycin-resistant *Vibrio* spp. that might occur in the environment would not be detected. The identity of *V. campbellii*, expressing GFP, was confirmed by fluorescence microscopy. The injection dose was plated for each shrimp as a positive control and also for use in the standard curve in the real-time PCR analysis described above.

### Statistical analysis

The effects of time after injection, tissue, and gas level on the total number of intact and culturable *V. campbellii* in the four tissues were analyzed using separate three-way analyses of variance (ANOVA). Using the experiment-wide three-way ANOVA instead of a series of individual tests minimized Type I error, which remained at 0.05. Time after injection, tissue, and gas level were treated as fixed effects, whereas individual shrimp were treated as a random effect to adjust for any variances among animals. The tests were modeled using R, a language for statistical computing (R Foundation for Statistical Computing, 2004). The use of R, and 'lme' within R, which differs from the normal least squares approach, allowed us to accurately estimate the parameters and significance of this unbalanced, mixed model. To meet assumptions of equal variance and normality, the weight-specific number of *V. campbellii*, either intact or culturable, was log-transformed, while the percentage of culturable *V. campbellii* was arc-sin square-root-transformed. SigmaStat 3.0 software (SPSS Inc.) was used to perform *post hoc* pairwise multiple comparisons on significant effects and interactions using the Holm-Sidak method (HS).

## Results

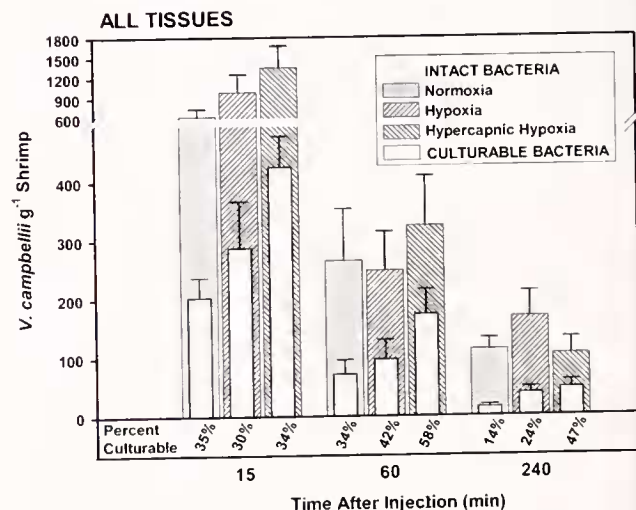
### Experiment-wide three-way analysis of variance

Injected bacteria that remained sufficiently intact to retain the pMSB6 plasmid were quantified by real-time PCR using primers directed against the plasmid kanR gene. The real-time PCR data were analyzed by a mixed-model three-way ANOVA to determine whether the numbers of intact bacteria in the target tissues of injected shrimp were significantly affected by each of three major factors: time after injection, tissue type, and treatment (normoxia, hypoxia, or hypercapnic hypoxia). By this analysis, time after injection and tissue type each had significant effects on the number of *Vibrio campbellii* in the tissues of *Litopenaeus vannamei* ( $P < 0.0001$ , degrees of freedom [df] = 229, for each factor). The effect of treatment on the accumulation of intact *V. campbellii* depended on the tissue type; that is, there was a significant interaction between treatment and tissue type ( $P < 0.0001$ , df = 229).

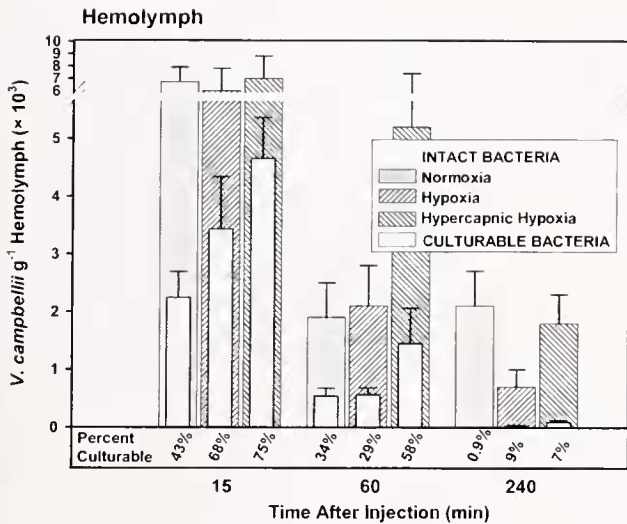
The number of culturable bacteria in the target tissues of injected shrimp represented a subset of intact bacteria that remained viable, could grow on selective medium, and retained the pMSB6 plasmid. A three-way ANOVA indicated that each of the three factors (time after injection, tissue type, and treatment) had significant effects on the number of culturable *V. campbellii* ( $P < 0.001$ , df = 235) as well as on the percentage of intact bacteria that were culturable ( $P < 0.001$ , df = 229). There was, however, a

significant interaction between time and tissue type ( $P < 0.0001$ , df = 229) and between all three factors ( $P = 0.0085$ , df = 229), meaning that the effect of time differed within different tissues and within different tissues and treatments, respectively.

In subsequent analyses, the effects of time and treatment on the localization and elimination of bacteria were examined in two ways. First, to provide an overall picture of what happened to the total bacterial dose injected into shrimp, we calculated the total number of intact bacteria, culturable bacteria, and percent culturable bacteria in each of the tested tissues at each time point, then divided those values by the weight of the shrimp. Since the bacterial dose was weight-adjusted, this value — total bacteria in all tissues summed per gram of shrimp (Fig. 1) — could be compared among shrimp, regardless of the animal weight. Then, changes in bacterial numbers within a single tissue type were calculated as a function of treatment, yielding the number of intact bacteria, culturable bacteria, and percent culturable bacteria per gram tissue for each tissue type and each time point (Figs. 2–4). Significant effects of time and treatment, as well as the interaction of time and treatment, in the tested tissues (total and individual tissues) and in the hemolymph, were separately tested for significance by two-way ANOVA (significance level < 0.05). *Post hoc* pairwise multiple comparisons on significant effects and interactions were tested by the Holm-Sidak method (HS).



**Figure 1.** Mean total numbers ( $\pm$  SEM) of intact (shaded bars) and culturable (open bars) *Vibrio campbellii* in all tested tissues (gills, hepatopancreas, heart, and lymphoid organ) of *Litopenaeus vannamei* as a function of treatment (normoxia, hypoxia, hypercapnic hypoxia) and time after injection of  $2 \times 10^4$  *V. campbellii*  $g^{-1}$  shrimp. The mean percentages of the intact *V. campbellii* that were culturable are reported on the x-axis below each respective bar. See the text and Tables 2 and 3 for detailed comparisons and statistical analyses.

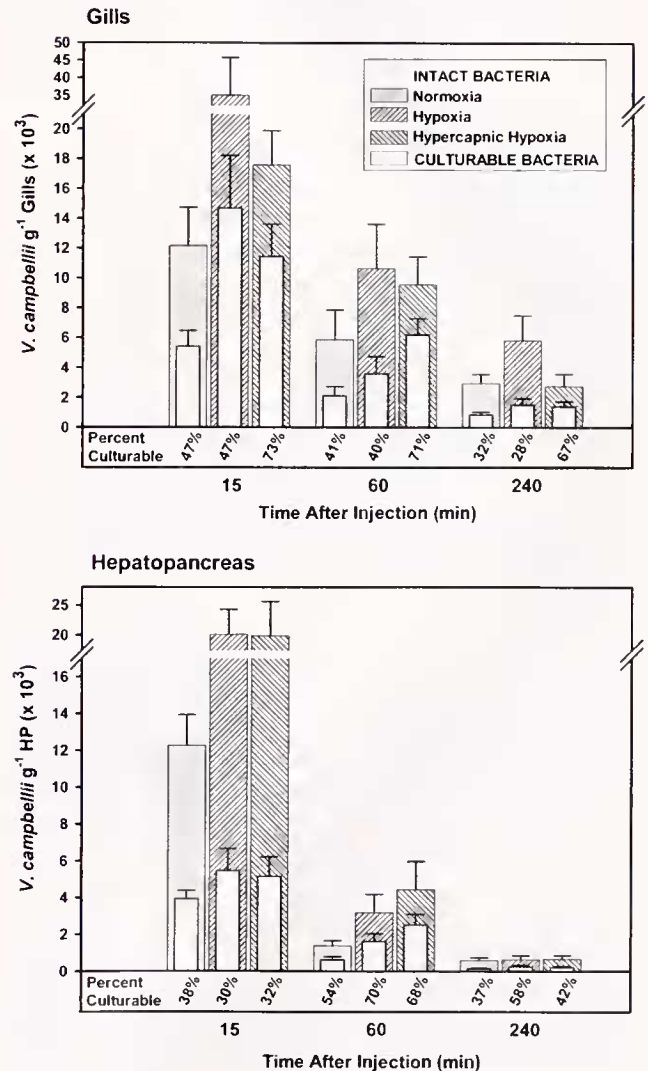


**Figure 2.** Mean number ( $\pm$  SEM) of intact (shaded bars) and culturable (open bars) *Vibrio campbellii* in the hemolymph of *Litopenaeus vannamei* 15, 60, and 240 min after injection of  $2 \times 10^3$  *V. campbellii* g<sup>-1</sup> shrimp. The mean percentages of the intact *V. campbellii* that were culturable are reported on the x-axis, below each respective bar. See the text and Tables 2 and 3 for detailed comparisons and statistical analyses.

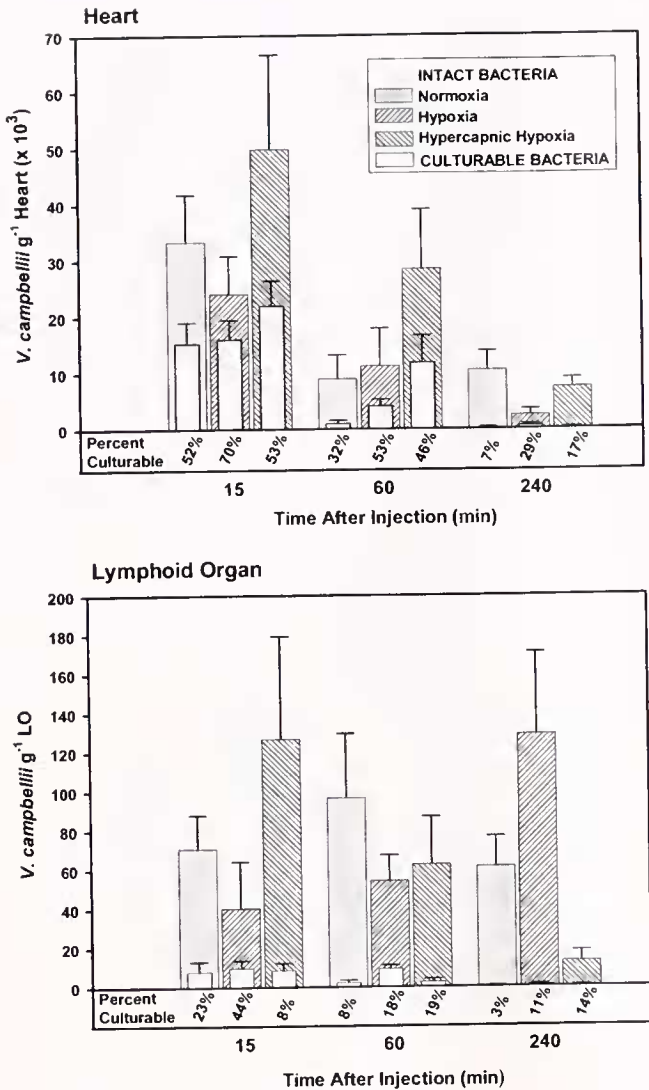
#### Effect of time on all tissues

For significant effects of time (ANOVA  $P < 0.05$ ),  $P$  values and corresponding degrees of freedom are given in Table 2. The average numbers ( $\pm$  SEM) of intact bacteria in all tissues summed (gills + hepatopancreas + heart + lymphoid organ) in *Vibrio*-injected animals are presented as shaded bars for each treatment group (normoxia, hypoxia, hypercapnic hypoxia) as a function of time (Fig. 1). Analysis by two-way ANOVA revealed a significant effect of time across all treatments on the number of intact bacteria in all tissues summed ( $P < 0.001$ , as shown in Table 2). Similarly, there was a significant effect of time on the number of intact bacteria in the hemolymph, as well as individually in the gills, the hepatopancreas, and the heart, across all treatments (Figs. 2, 3, 4; Table 2). In contrast, there was no significant effect of time across all treatments on the number of intact *V. campbellii* in the lymphoid organ (Fig. 4), but the two-way ANOVA revealed a significant interaction of time and treatment. *Post hoc* analysis showed a significant effect of time with exposure to hypercapnic hypoxia (HS,  $P = 0.002$ , Table 3), but not to hypoxia or normoxia, on intact bacteria in the lymphoid organ. The *post hoc* analysis does not reveal the direction of change, but data in Figure 4 indicate a decrease in intact bacteria in the lymphoid organ over time. The number of culturable *V. campbellii* also decreased over time, regardless of treatment, in all tissues summed (Fig. 1), as well as in the hemolymph and individual tissues, including the lymphoid organ (Figs. 2–4).

In all tissues summed and individually in the hemolymph, the heart, and the lymphoid organ, the percentage of intact bacteria that were culturable decreased over time (Figs. 1, 2, 4; Table 2). In contrast, the percentage of intact *V. campbellii* that could be cultured from the gills did not change over time, but in the hepatopancreas this value did increase with time (Fig. 3; Table 2). Pairwise analysis showed that the increase in the hepatopancreas occurred between 15 and 60 min after injection (HS,  $P = 0.005$ ). The effect of time on the percentage of intact bacteria that were culturable did not significantly vary as a function of treatment (normoxia,



**Figure 3.** Mean number ( $\pm$  SEM) of intact (shaded bars) and culturable (open bars) *Vibrio campbellii* in the gills and the hepatopancreas (HP) of *Litopenaeus vannamei* per unit tissue weight at 15, 60, and 240 min after injection of  $2 \times 10^3$  *V. campbellii* g<sup>-1</sup> shrimp. The mean percentages of the intact *V. campbellii* that were culturable are reported on the x-axis, below each respective bar. See the text and Tables 2 and 3 for detailed comparisons and statistical analyses.



**Figure 4.** Mean number ( $\pm$  SEM) of intact (shaded bars) and culturable (open bars) *Vibrio campbellii* in the heart and the lymphoid organ (LO) of *Litopenaeus vannamei* per unit tissue weight at 15, 60, and 240 min after injection of  $2 \times 10^4$  *V. campbellii*  $g^{-1}$  shrimp. The mean percentages of the intact *V. campbellii* that were culturable are reported on the x-axis, below each respective bar. See the text and Tables 2 and 3 for detailed comparisons and statistical analyses.

hypoxia, hypercapnic hypoxia) — neither in all tissues summed nor in individual tissues.

#### Effect of treatment on all tissues and hemolymph

As indicated above (Experiment-wide three-way ANOVA), treatment as a variable and independent of time and tissue type had significant effects on the number of culturable *V. campbellii* as well as on the percentage of intact bacteria that were culturable. The effect of treatment on the accumulation of intact bacteria depended on the tissue type; therefore, treatment effects are presented sepa-

rately below for (1) all tissues summed and hemolymph, (2) gills and hepatopancreas, and (3) heart and lymphoid organ.

For significant effects across treatments (ANOVA  $P < 0.05$ ),  $P$  values and corresponding degrees of freedom are given in the text below, while the HS  $P$  values from *post hoc* pairwise tests of hypoxia or hypercapnic hypoxia versus normoxia are provided in Table 3. The total numbers of intact *V. campbellii* recovered from all of the tissues (gills + hepatopancreas + heart + lymphoid organ) did not significantly vary with treatment; however, treatment significantly affected the number and percentage of culturable bacteria (ANOVA  $P < 0.003$ ,  $P < 0.001$ , respectively;  $df = 53$ ) (Fig. 1). In pairwise comparisons with normoxia, hypercapnic hypoxia, but not hypoxia, increased both the number and the percentage of culturable bacteria (Table 3).

The number of intact *V. campbellii* recovered from the hemolymph did not vary significantly with treatment (Fig. 2). Treatment significantly impacted both the number and the percentage of culturable bacteria in the hemolymph ( $P = < 0.001$ ,  $df = 69$  and  $P = 0.034$ ,  $df = 61$ , respectively), although the effect varied among the treatments. Shrimp exposed to hypoxia had a greater number, but not a greater percentage of culturable *V. campbellii* in the hemolymph, while the animals in hypercapnic hypoxia had both a greater number and a greater percentage of intact bacteria that could be cultured in the hemolymph as compared to normoxic controls (Fig. 2; Table 3).

#### Effect of treatment on gills and hepatopancreas

Treatment had a significant effect on the numbers of intact and culturable *V. campbellii* in the gills ( $P = 0.013$ ,  $P = 0.002$ , respectively;  $df = 71$ ) and hepatopancreas ( $P < 0.001$ ,  $df = 70$  for both tests) and on the percent culturable bacteria in the gills ( $P < 0.001$ ,  $df = 71$ ). Exposure to hypoxia, but not to hypercapnic hypoxia, increased the number of intact bacteria in the gill, as compared to normoxia. Both hypoxia and hypercapnic hypoxia increased the number of intact *V. campbellii* in the shrimp hepatopancreas, as compared to normoxia. In both the gills and the hepatopancreas, there were also significantly more culturable *V. campbellii* in shrimp exposed to hypoxia and hypercapnic hypoxia than in normoxic animals. The percentage of culturable *V. campbellii* in the gills of shrimp exposed to hypercapnic hypoxia, but not to hypoxia, was higher than in animals held in normoxia. When compared to normoxia, neither hypoxia nor hypercapnic hypoxia had a significant effect on the percentage of culturable *V. campbellii* in the shrimp hepatopancreas (Fig. 3; Table 3).

#### Effect of treatment on heart and lymphoid organ

Neither hypoxia nor hypercapnic hypoxia had a significant effect on the number of intact, culturable, or percent culturable *V. campbellii* in the heart (Fig. 4; Table 3).

Table 2

Results of ANOVA for the significant effects of time on *Vibrio campbellii* (intact, culturable, or percent culturable bacteria) in tested tissues and hemolymph across all treatments

Tissue	Direction of change in bacteria with time and ANOVA <i>P</i> values (degrees of freedom)		
	Intact bacteria	Culturable bacteria	% Culturable bacteria
All tissues (gills + hepatopancreas + heart + lymphoid organ)	↓ <i>P</i> = 0.001 (53)	↓ <i>P</i> = 0.001 (53)	↓ <i>P</i> = 0.022 (53)
Hemolymph	↓ <i>P</i> = 0.001 (61)	↓ <i>P</i> = 0.001 (69)	↓ <i>P</i> = 0.001 (61)
Gills	↓ <i>P</i> = 0.001 (71)	↓ <i>P</i> = 0.001 (71)	NS (71)
Hepatopancreas	↓ <i>P</i> = 0.001 (70)	↓ <i>P</i> = 0.001 (70)	↑* <i>P</i> = 0.017 (70)
Heart	↓ <i>P</i> = 0.001 (61)	↓ <i>P</i> = 0.001 (61)	↓ <i>P</i> = 0.001 (61)
Lymphoid organ	NS** (58)	↓ <i>P</i> = 0.002 (70)	↓** <i>P</i> = 0.028 (58)

↓ and ↑ indicate a decrease or increase, respectively, in the direction of change of the variable with time. NS = no significant effect of time (ANOVA *P* > 0.05).

\* Increase between 15 and 60 min (Holm-Sidak method, *P* = 0.005).

\*\* Significant interaction with treatment; see Table 3 for the results of *post hoc* tests.

As mentioned previously, the lymphoid organ was the only tissue in which there was a significant interaction between treatment and time after injection for a measure of *V. campbellii* quantified in this study. Specifically, the effect of either hypoxia or hypercapnic hypoxia on the number of intact *V. campbellii* in the lymphoid organ depended on the

time after injection (*P* = 0.013, *df* = 60 for the interaction). Only at 240 min were fewer intact *V. campbellii* detected in the lymphoid organ of shrimp exposed to hypercapnic hypoxia compared to shrimp exposed to either hypoxia or normoxia (Fig. 4). As compared to normoxia, neither hypoxia nor hypercapnic hypoxia had a significant effect on the

Table 3

Pairwise multiple comparison analysis of *Vibrio campbellii* (intact, culturable, or percent culturable bacteria) in body compartments of shrimp testing the effects of hypoxia or hypercapnic hypoxia versus normoxia.

Tissue	Treatment	Difference from normoxia, Holm-Sidak <i>P</i> values ( <i>n</i> )		
		Intact bacteria	Culturable bacteria	% Culturable bacteria
All tissues (gill + hepatopancreas + heart + lymphoid organ)	Normoxia		NS (22)	NS (22)
	Hypoxia		NS (15)	NS (15)
	Hypercapnic hypoxia		<i>P</i> < 0.001 (17)	<i>P</i> < 0.001 (17)
Hemolymph	Normoxia		NS (30)	NS (27)
	Hypoxia		<i>P</i> = 0.008 (21)	NS (18)
	Hypercapnic hypoxia		<i>P</i> < 0.001 (19)	<i>P</i> = 0.011 (17)
Gills	Normoxia	NS (30)	NS (30)	NS (30)
	Hypoxia	<i>P</i> = 0.003 (21)	<i>P</i> = 0.004 (21)	NS (21)
	Hypercapnic hypoxia	NS (21)	<i>P</i> = 0.002 (21)	<i>P</i> < 0.001 (21)
Hepatopancreas	Normoxia	NS (30)	NS (30)	NS (30)
	Hypoxia (21, all tests)	<i>P</i> = 0.043 (21)	<i>P</i> = 0.010 (21)	NS (21)
	Hypercapnic hypoxia	<i>P</i> = 0.013 (20)	<i>P</i> = 0.004 (20)	NS (20)
Heart	Normoxia			
	Hypoxia			
	Hypercapnic hypoxia			
Lymphoid organ	Normoxia	NS (26)		NS (26)
	Hypoxia	NS (17)		<i>P</i> = 0.013 (17)
	Hypercapnic hypoxia	NS* (18)		NS (18)

The Holm-Sidak method for multiple comparisons (significance level = 0.05) was performed only on data sets for which two-way ANOVA showed a significant effect of treatment or a significant interaction with time (*P* < 0.05). Shaded areas = no significant treatment effect and no interaction of treatment with time as determined by two-way ANOVA. NS = no significant effect by the Holm-Sidak method (*P* > 0.05). Values in parentheses are numbers of samples tested from each treatment group.

\* Significant interaction between time and treatment. At 240 min, ANOVA, *P* < 0.014, *df* = 60.

number of culturable *V. campbellii* recovered from the lymphoid organ; however, exposure to hypoxia significantly increased the percentage of culturable *V. campbellii* in this tissue (Fig 4; Table 3).

### Discussion

In the present study, we demonstrate that hypercapnic hypoxia and, to a lesser extent, hypoxia inhibit the overall ability of the shrimp *Litopenaeus vannamei* to reduce the numbers of culturable *Vibrio campbellii* in its hemolymph and tissues during the first 4 h after injection. Across all timepoints examined, hypoxia and hypercapnic hypoxia did not significantly alter the numbers of intact bacteria in whole animals (all tissues summed), as compared to normoxia. Examination of individual tissues, however, revealed significant treatment effects on the numbers of intact bacteria in gills, hepatopancreas, and lymphoid organ (Table 3). Where significant effects of hypoxia, hypercapnic hypoxia, or both were detected, there were increases in gills and hepatopancreas and decreases in the lymphoid organ (Table 3). These results are consistent with a shift in the distribution of bacteria in the major tissues away from the lymphoid organ towards the gills and the hepatopancreas.

Our data confirm the results of previous studies showing that both hypoxia and hypercapnic hypoxia impair the removal of live, culturable bacteria from the hemolymph of decapod crustaceans (Direkbusarakom and Danayadol, 1998; Cheng *et al.*, 2002; Holman *et al.*, 2004). To our knowledge, this study provides the first demonstration that hypoxia and hypercapnic hypoxia suppress bacteriostasis but do not impair the physical removal of intact bacteria from the hemolymph.

Although numerous mechanisms are likely to be involved in the bacteriostasis of *V. campbellii*, the present data suggest that at least some of these mechanisms are inhibited by hypoxia and hypercapnic hypoxia. Low dissolved oxygen and pH suppress several specific immune functions, such as phagocytosis, the production of ROS, and the prophenoloxidase cascade (Smith, 1991; Muñoz *et al.*, 2000; Bachère *et al.*, 2004; Cerenius and Söderhäll, 2004). Antimicrobial peptides (Bachère *et al.*, 2004) as well as the *in vivo* formation of hemocyte-bacterial nodules (Martin *et al.*, 1998) are believed to contribute to suppressing the growth of bacterial pathogens in shrimp. The sensitivities of the antimicrobial peptides and nodule formation to hypoxia and hypercapnic hypoxia have yet to be assessed.

Recent evidence suggests that, in penaeid shrimp, the lymphoid organ is a major site of active uptake of bacteria from the hemolymph (van de Braak *et al.*, 2002; Burgents *et al.*, 2005). After bacteria are injected, hemocytes migrate from the hemolymph to the lymphoid organ, where they phagocytose invading bacteria (van de Braak *et al.*, 2002). Hypoxia and hypercapnic hypoxia are believed to decrease

the activity of shrimp hemocytes, as measured by a decrease in ROS production (Le Moullac *et al.*, 1998) and phagocytosis (Cheng *et al.*, 2002), which consequently would reduce the accumulation of bacteria at sites of active hemocyte uptake. The latter findings are consistent with the results of the present study — higher numbers of culturable *V. campbellii* remaining in the hemolymph and changes in the lymphoid organ — that indicate both reduced accumulation of intact bacteria under hypercapnic hypoxia and reduced bacteriostatic activity in hypoxia. Both the hepatopancreas and the gills are target tissues of *Vibrio* infection in penaeid shrimp (Lightner, 1996). The elevated number of culturable bacteria circulating in the hemolymph may allow additional time for these pathogens to accumulate in the hepatopancreas and the gills, explaining the elevated mortality rates associated with exposure to hypoxia and hypercapnic hypoxia.

In decapod crustaceans, the gills are a major site of bacterial accumulation under normoxic conditions (Smith and Ratcliffe, 1980, 1981; White and Ratcliffe, 1982; Martin *et al.*, 1993; Alday-Sanz, 2002). The results of the present study suggest that, if the gills actively take up bacteria from the hemolymph, then the mechanisms responsible for this sequestration are not inhibited by either hypoxia or hypercapnic hypoxia. Instead, significantly more intact *V. campbellii* per unit tissue weight were recovered from the gills after exposure to hypoxia compared to normoxia, and significantly more culturable *V. campbellii* per unit tissue weight were recovered from the gills after exposure to either hypoxia or hypercapnic hypoxia. Since the gills are a target for the pathogenic effects of vibriosis (Lightner, 1996), the enhanced accumulation of *V. campbellii* we observed in the gills provides a logical explanation for the increased pathogenicity of *Vibrio* spp. in shrimp exposed to hypoxia and hypercapnic hypoxia (Le Moullac *et al.*, 1998; Mikulski *et al.*, 2000).

It is possible that the observed increase in bacterial accumulation at the gills is mediated by changes in virulence factors expressed by the bacteria or by gill-surface receptors that mediate the adherence of *Vibrio* spp. to their target tissues. Alternatively, an increase in bacterial accumulation at the gills might be related to hemodynamic changes associated with responses to hypoxia. Decapod crustaceans generally increase cardiac output in response to environmental hypoxia (reviewed by McMahon, 2001). The elevated perfusion of the gills improves oxygenation of the hemolymph but, as suggested by Martin *et al.* (1993), it may also provide more opportunity for the entrapment of nodules formed by hemocytes and bacteria. Furthermore, elevated hydrostatic pressures driving the hemolymph through the gills cause the hemolymph spaces within the gills to expand (McMahon and Burnett, 1990), a property known as compliance. Simultaneously, hydrostatic pressures in the chamber surrounding the gills, which are already negative, be-



come more negative with increased activity of the water pumping mechanism (reviewed by McMahon, 2001). Thus, the increased positive pressure in the hemolymph space within the gill and the increased negative pressure outside the gill both act to lower vascular resistance, resulting in a preferential shunting of hemolymph through the gill. In general, hemolymph flow during hypoxia appears to be shunted away from the viscera (Airriess and McMahon, 1994).

In all tissues combined (gills + hepatopancreas + heart + lymphoid organ) and in the hemolymph, hypercapnic hypoxia had a greater impact than hypoxia alone on the number and percentage of culturable bacteria (Table 3). Most studies investigating the effects of hypoxia on the immune system have ignored the associated increase in CO<sub>2</sub>, or hypercapnia, which causes a decrease in water pH. Although not previously documented in shrimp, a decrease in pH has an independent and negative effect on the *in vitro* production of ROS by oyster hemocytes (Boyd and Burnett, 1999) as well as a negative effect on the bacteriostatic activity of fish phagocytes (Boleza *et al.*, 2001). The data presented here further document the importance of considering both dissolved oxygen levels and pH when assessing the immunological competence of marine organisms.

The results of the present study suggest that the decreased resistance to bacterial pathogens associated with exposure to hypoxia and hypercapnic hypoxia at levels that are normally sublethal may be partly due to decreases in the accumulation of bacteria and the bacteriostatic activity in the lymphoid organ, a tissue that converts a large percentage of accumulated bacteria from a culturable to a non-culturable state (Burgents *et al.*, 2005). Exposure to hypoxia and hypercapnic hypoxia also increases the number of culturable bacteria that remain in the hemolymph over the first 4 hours after exposure to the pathogen. The increased presence of intact and culturable bacteria in the hemolymph is consistent with the greater uptake of bacteria in the gills and hepatopancreas and the increased exposure of these target organs to *V. campbellii* under conditions of hypoxia and hypercapnic hypoxia. It is clear that these levels of hypoxia and hypercapnic hypoxia have an effect on immune defense mechanisms that results in a decreased resistance to invading pathogens.

#### Acknowledgments

This paper was based upon work supported by the National Science Foundation under Grant No. IBN-0212921 to Dr. Karen Burnett and Dr. Lou Burnett. We wish to acknowledge Dr. Eric Stabb for providing the transformed *V. campbellii* strain and for real-time PCR technical support, Dr. Erin Burge for technical advice, and Dr. Allan Strand for statistical analysis. Contribution # 271 of the Grice Marine Laboratory.

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